CHAPTER 7

DNA Cleavage with N4Py Iron Complexes

7.1 Structure of Bleomycin

Bleomycins (BLM's)[1] are a family of glycopeptide antibiotics, which are clinically used in cancer treatment. Their cytotoxicity is generally thought to be related to the ability to bind and oxidatively cleave DNA.[2] For their activity they depend on an iron(II) atom in combination with O_2 and an electron source as cofactors.
The various naturally occurring bleomycins have a very similar structure (figure 1). They only differ in the nature of the positively charged R group at the terminus. In the case of bleomycin A2, which is the major constituent of natural bleomycin, this is a dimethylsulfonium group. Three principal domains can be distinguished.

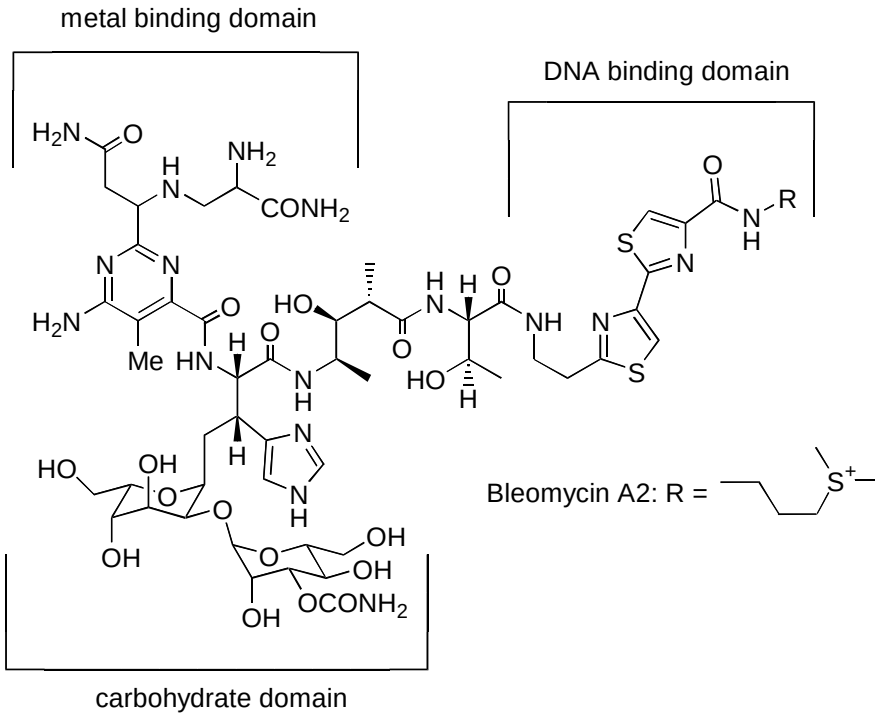


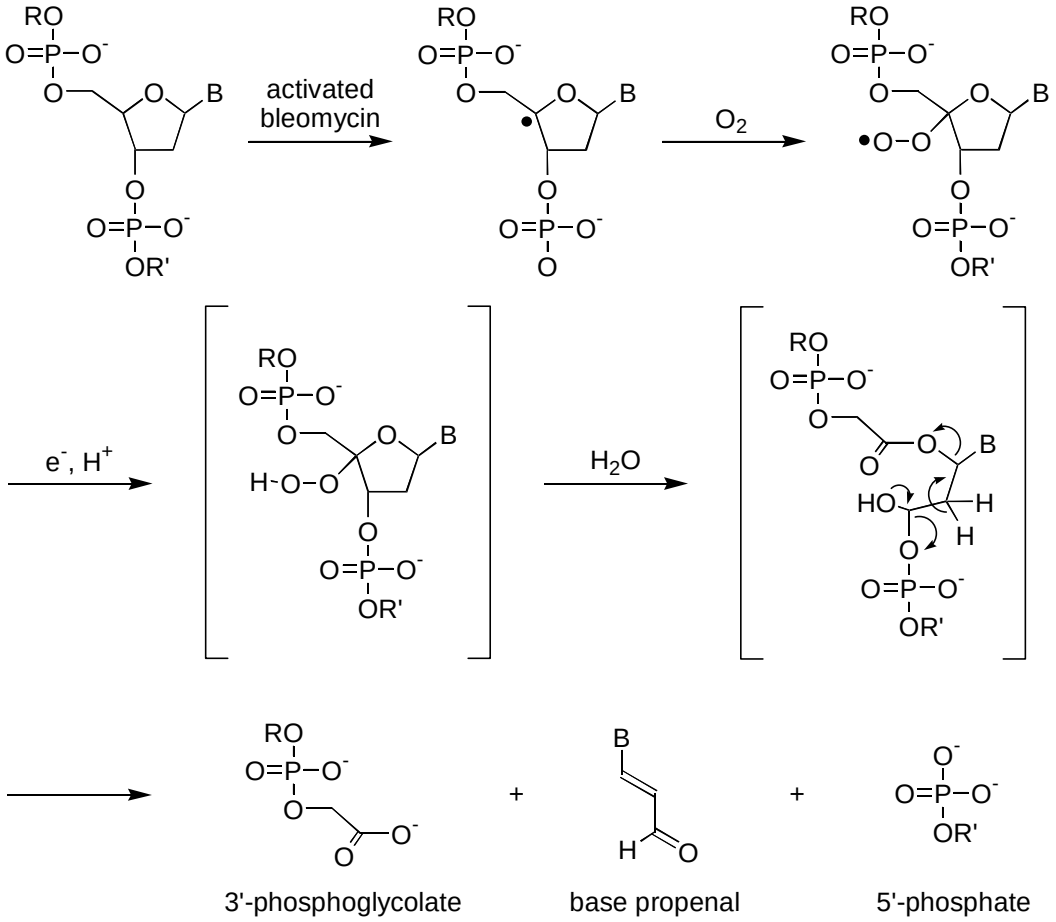
Figure 1. *Structure of Bleomycin.*

First of all there is the metal binding domain where iron can be bound by five nitrogen donor atoms.[3] At the iron centre O_2 is bound to give the active complex responsible for DNA strand cleavage.[2,4] Furthermore, the metal binding domain seems to be primarily responsible for the observed sequence selectivity at GpC(5'→3') and GpT(5'→3')[5] in DNA cleavage, with an important role for the pyrimidine group of BLM.[6] Secondly, there is the DNA binding domain consisting of a bithiazole moiety attached to the metal binding domain via a short peptide spacer. DNA binding is believed to occur via intercalation of the bithiazole moiety into the minor groove DNA strand, with the possible aid of electrostatic interactions of the usually positively charged R group, *e.g.* a sulfonium ion, with the negatively charged

phosphate backbone of the DNA.[7] The bithiazole unit itself is only responsible for binding affinity and not binding selectivity, but the peptide linker has been implicated to be involved in sequence specific binding.[8] Furthermore, it was demonstrated that the DNA binding domain in combination with the peptide linker is essential for double strand cleavage (*vide infra*).[8,9]
Finally, a disaccharide moiety is present, whose function is not completely understood. It is proposed to be responsible for selective accumulation of bleomycin in some cancer cells. However, the disaccharide moiety is not required for DNA cleavage.[10]

7.2 Mechanism of DNA Cleavage

Fe-BLM causes both single strand breaks (ssb) and double strand breaks (dsb) in DNA. The generally accepted mechanistic proposal for bleomycin action involves binding to DNA followed by reaction of the Fe^{II} centre with O_2 to form activated bleomycin, the last detectable intermediate prior to DNA cleavage.[11] Whether activated bleomycin is the active species or the precursor to the active species is a question still unanswered.[12]

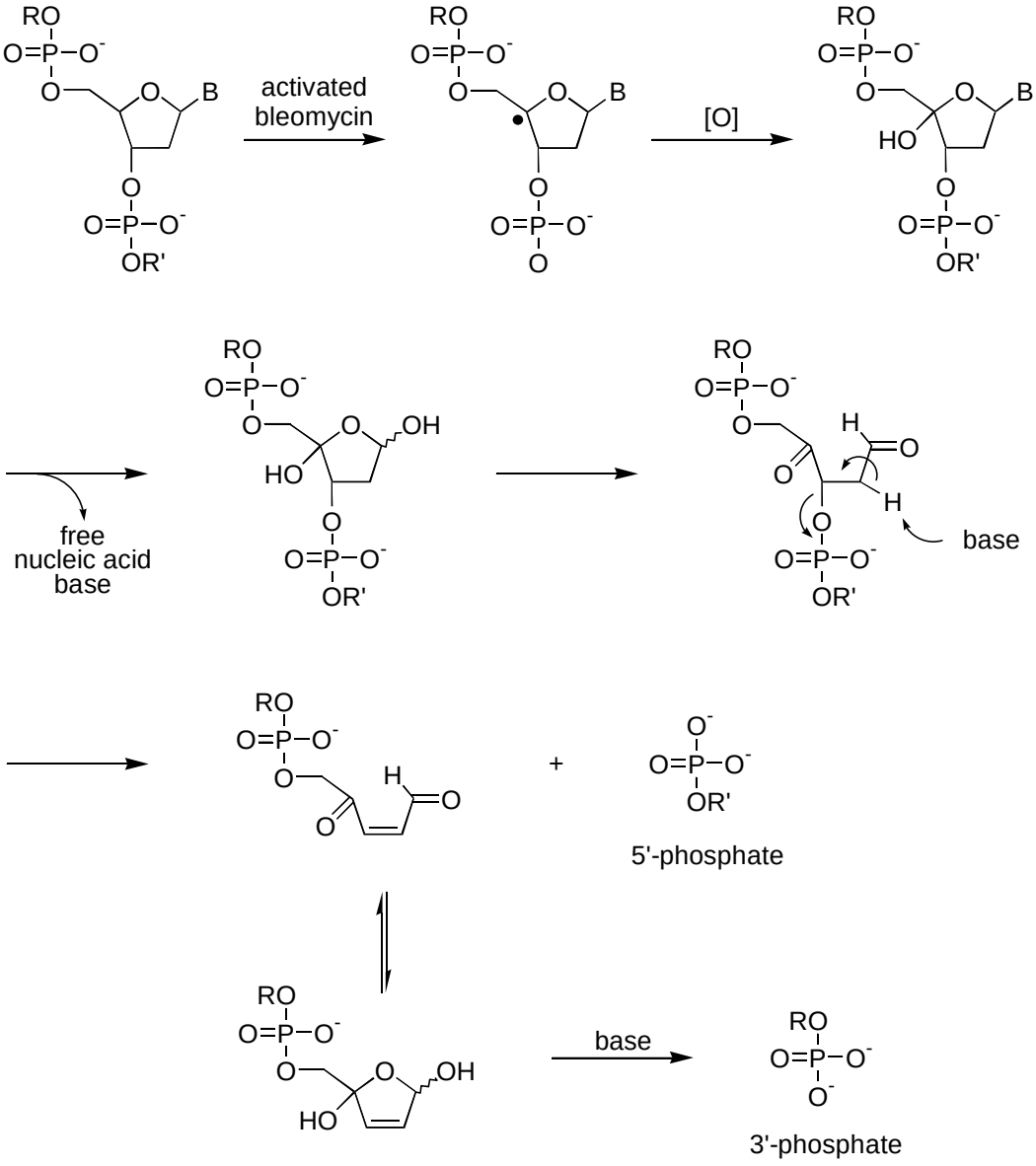


Scheme 1. *Mechanism of DNA cleavage by Fe-BLM under aerobic conditions.*

Hydrogen abstraction at the C4' position of the deoxyribose ring results in the formation of a C4' based radical intermediate. This step is rate limiting as was indicated by the large

deuterium and tritium isotope effects (k_H/k_D = 2-7, k_H/k_T = 7-11).[2d,13] The fate of this intermediate is determined by two subsequent pathways, one that requires O_2 and one that does not.[2,14] Under aerobic conditions the carbon radical intermediate reacts with O_2 to form a peroxyl radical, which may undergo a Criegee-like rearrangement (scheme 1).[15] The characteristic products obtained via this route are DNA fragments with 3'-phosphoglycolate termini, base propenal and DNA fragments with 5'-phosphate termini.[2d,13a,14b,16] Base propenal and the 3'-phosphoglycolate terminated strand are characteristic products for oxidation at C4'.[14a]

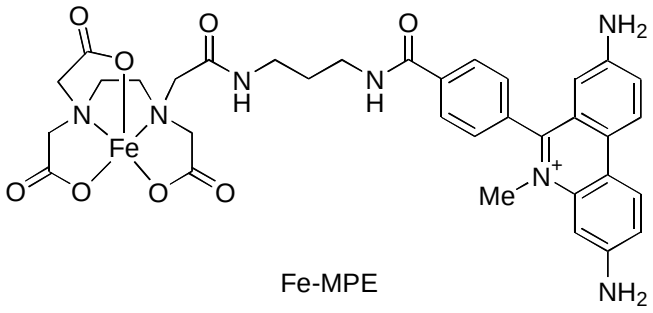
Under oxygen deficient conditions the 4'-deoxyribosyl radical is oxidized to produce a 4' hydroxylated species (Scheme 2). Base release followed by β-elimination gives the 5'-phosphate terminated DNA strand and a 4'-keto-1'-aldehyde terminated strand. The latter is alkali labile and decomposes in the presence of base to a 3'-phosphate terminated strand.[14b,17] The ratio between these two mechanistic pathways is dependent on O_2 concentration.



Scheme 2. *Mechanism of DNA cleavage by Fe-BLM under O_2 deficient conditions.*

7.3 Synthetic Iron Containing DNA Cleaving Agents

A large variety of synthetic DNA cleaving agents using a number of metal cofactors have been reported in the literature.[14a,18] Synthetic iron complexes with DNA cleavage activity include iron-EDTA[19] and a few other iron complexes.[20] Introduction of a DNA binding or intercalating unit greatly enhances the cleavage activity of these synthetic systems. This principle was first demonstrated by Dervan and co-workers with the Fe-MPE system, consisting of an EDTA metal binding domain covalently attached via a short spacer to a methidium group, which intercalates into the minor groove of the DNA (figure 2).[21] Because of its ability to give non-selective DNA cleavage Fe-MPE has been used successfully in DNA footprinting studies.[22] The methidium group could also be replaced by more selective DNA binding groups like distamycin,[23] oligodeoxynucleotides[24] and hairpin polyamides,[25] which resulted in sequence selective DNA cleavage, *i.e.* cleavage at a specific base pair sequence.



Fe-MPE

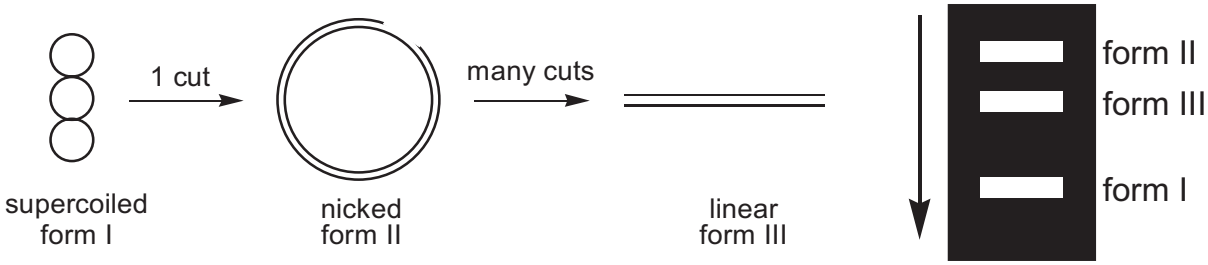
Figure 2. *Iron methidium propyl EDTA (Fe-MPE).*

This concept of covalently attaching a DNA intercalating/binding unit to a ligand has also been applied to other iron systems.[26] Many of these synthetic DNA cleaving complexes have to be prepared in situ, and almost all these systems use H_2O_2 or O_2 in combination with large excesses of reductants like dithiothreitol (DTT) or ascorbate (typically 1 mM). Especially the last feature may limit the scope for biological application at the cellular level.[27]
In the previous chapters the iron bleomycin model $[(N4Py)Fe(CH_3CN)](ClO_4)_2$ (**1**), where N4Py is a pentadentate N5 ligand like bleomycin, was described. Complex **1** forms a transient low-spin Fe(III)-OOH species that has properties resembling those of "activated bleomycin". Therefore its its DNA cleaving properties and those of an N4Py derivative with a tethered acridine intercalating moiety were investigated. With this strategy, a new biomimetic DNA cleaving agent that is effective with O_2 alone was prepared. The results of this study are described below.

7.4 DNA Cleavage: Principles

For DNA cleavage experiments Litmus29 plasmid DNA was used. Litmus29 is a high copy number ds-DNA plasmid with 2820 base pairs.[28] DNA cleavage was followed by monitoring the conversion of supercoiled (form I) DNA to give nicked- (form II) and linear DNA (form III) (scheme 3). Further cleavage will result in smaller DNA fragments, which are sometimes

referred to as degraded DNA. One single cut is sufficient to convert supercoiled to nicked DNA. However, to convert nicked to linear DNA requires cuts in opposite strands sufficiently near to each other, typically within 16 base pairs.[29] If cuts are farther apart the hydrogen bonding network of the DNA will hold the fragments together. With a random cleaving agents this means many cuts are required to convert nicked to linear DNA.



Scheme 3. *(a) Schematic representation of cleavage of supercoiled DNA. (b) Typical appearance of size exclusion gels found after DNA cleavage.*

A distinction is made between single strand cleavage and double strand cleavage. In a single strand cleavage reaction most supercoiled DNA is converted to nicked DNA before linear DNA is formed. An example of this class is Fe-MPE. A double strand cleaving agent is capable of cutting both strands either simultaneously or in rapid succession. This results in almost immediate formation of linear DNA. A typical example of this class is Fe-BLM.
Analysis of the DNA cleavage products is done by size exclusion gel electrophoresis. A schematic representation of a typical gel is shown in scheme 3b. Sometimes also a small band of not-nicked form II DNA (relaxed DNA) is found at the top of the gel. This is a side product, which comes from the DNA isolation.

7.5 DNA Cleavage with $[(N4Py)Fe(CH_3CN)](ClO_4)_2$

The DNA cleavage reactions were initiated by adding the metal complex to a buffered solution of Litmus 29 supercoiled plasmid DNA (0.1 μg/μL) and quenched by adding 10 μL of the solution to a solution of number III dye[30] and freezing with liquid nitrogen. Treatment of the DNA with 10 μM $[Fe(N4Py)(CH_3CN)](ClO_4)_2$ (**1**) in the presence of air at pH 8 and 37 °C resulted in the near immediate (quenching within 20 s after addition of the metal complex) formation of nicked DNA (28 %) (figure 3, table 1). This is a quite remarkable observation, since the cleavage chemistry was carried out in the absence of H_2O_2 or excess reductant, in contrast to the case with Fe(MPE). Monitoring the cleavage in time shows that after 18 h virtually all supercoiled DNA has disappeared and a small amount of linear DNA is formed. This reaction pattern is typical for a single strand cleavage reaction.

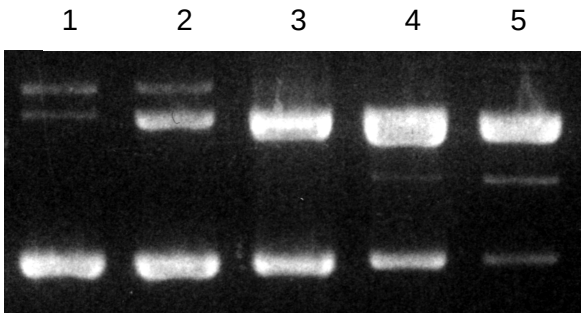


Figure 3. *Gel of DNA cleavage with **1** at 10 μM concentration. Lane 1, control; lane 2, t = 0 h; lane 3, t = 1 h; lane 4, t = 4.5 h; lane 5, t = 18 h.*

Table 1, *DNA cleavage with **1** at 10 μM concentration.*[a]

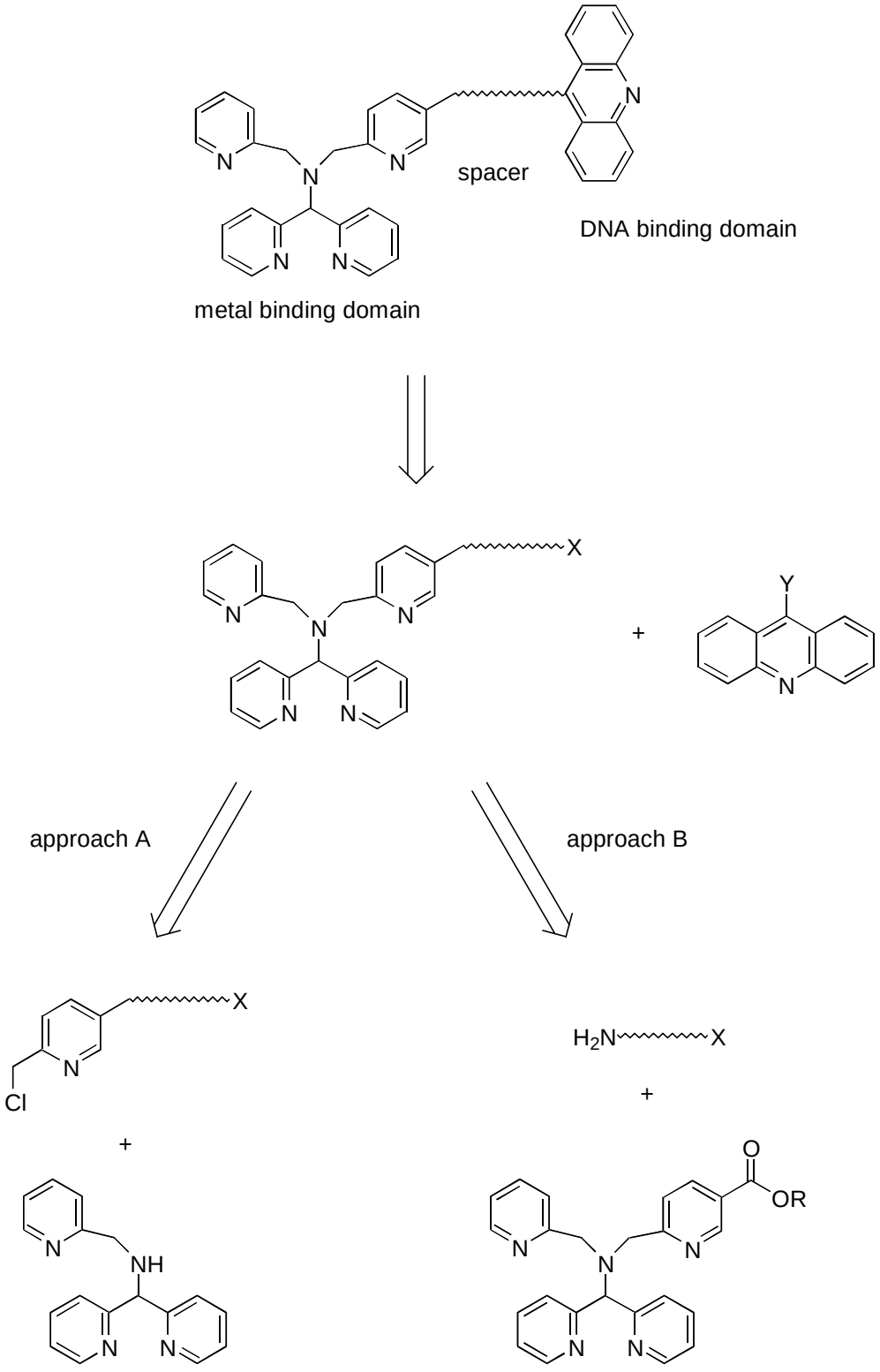
lane	time (h)	% form I[b]	% form II	% form III
2	0	72	28	0
3	1	40	60	0
4	4.5	27	72	1
5	18	9	85	6

(a) Results not corrected for reduced ethidium bromide uptake capability of form I. (b) Corrected for not-nicked form II DNA.

7.6 N4Py with a Covalently Attached Acridine: Concept and Retrosynthesis

To further increase the DNA cleaving ability of these complexes the design principles of bleomycin and MPE were followed. Introduction of a covalently attached DNA binding unit should increase the affinity of the complex for DNA and thus increase the DNA cleavage activity (scheme 4). As DNA binding unit an acridine moiety was chosen. Acridines intercalate into the minor groove of the DNA strand with a high binding constant ($K = 4.0 \times 10^5\ M^{-1}$ for 9-amino acridine)[31] and very little sequence specificity.[26] The acridine is attached to the ligand via a spacer, whose nature and length were mainly chosen for reasons of synthetic convenience. The spacer is attached to the ligand at the 5 position of one of the pyridylmethyl groups. This position was chosen for synthetic reasons.[32] Furthermore, introduction of groups at the 6 position of the pyridines may lead to changes in the iron chemistry of the complex, which is undesirable.[33]

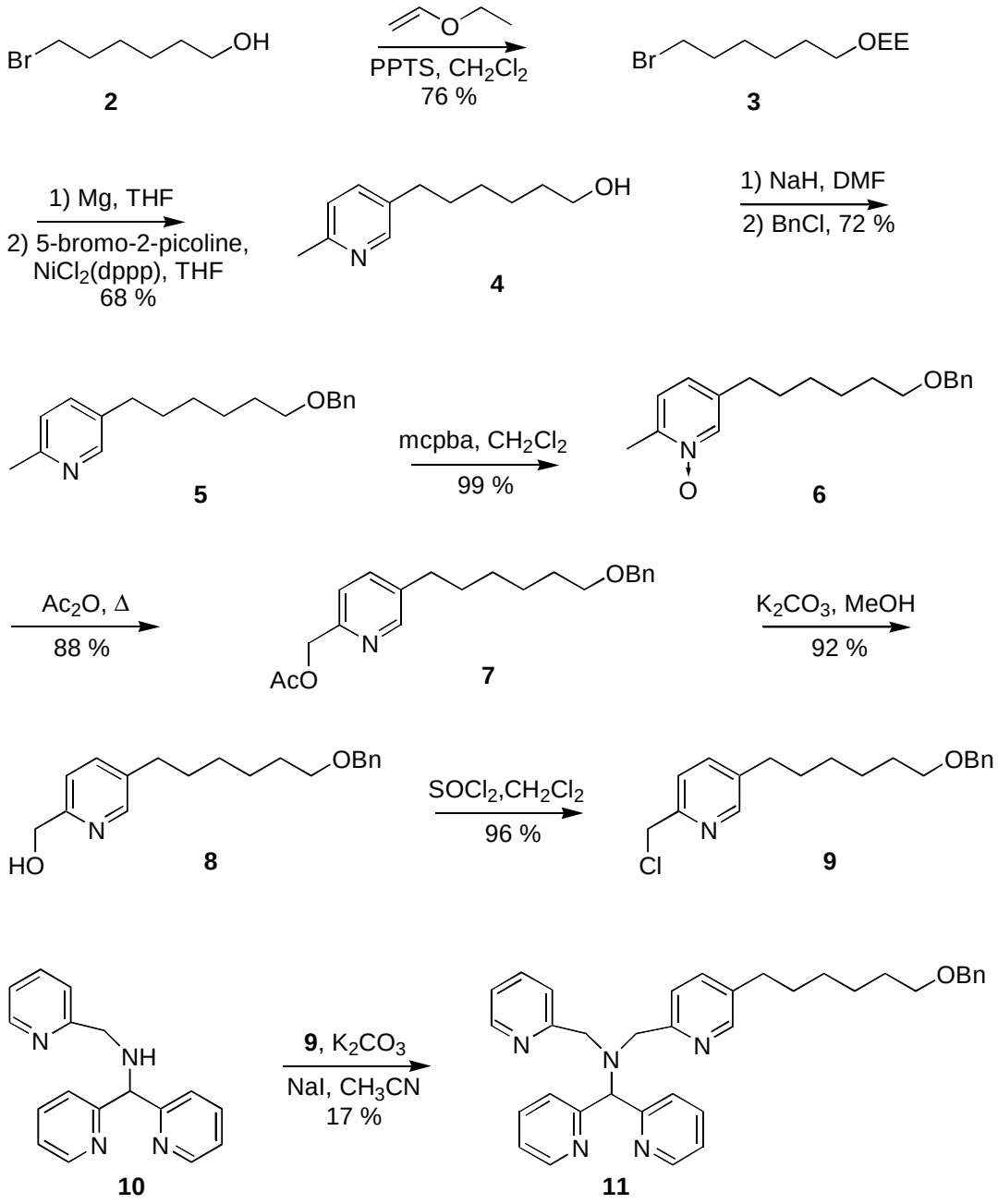
Two strategies have been followed. In both approaches the acridine is introduced in the final stage because acridines are not compatible with many synthetic conditions.[34] In approach A the spacer is first attached to a picoline derivative which is coupled with N3Py. In approach B first an ester functionalized N4Py part is prepared. The spacer is then introduced followed by coupling with the acridine.



Scheme 4. *Concept and retrosynthesis of an N4Py ligand with a covalently attached DNA binding moiety.*

7.7 Approach A

In this approach the spacer is first attached to 2-picoline via a $NiCl_2$(dppp) catalyzed Grignard cross coupling reaction of 5-bromo-2-picoline with ethoxy ethyl (EE) protected 6-hydroxy-hexane-1-magnesium bromide (scheme 5). After acidic workup deprotected **4** was obtained in reasonable yield. The hydroxy group was again protected as a benzyl ether, which is more robust. The picoline derivative **5** was then converted to the picolyl chloride derivative **9** using the standard procedure[32] of *N*-oxide formation, followed by reaction with acetic anhydride to give the acetate **7**, hydrolysis with K_2CO_3 in methanol and finally reaction with $SOCl_2$ to give the chloride **9**. These steps all proceed in excellent yields. Reaction of **9** with N3Py (**10**) gave the corresponding N4Py derivative **11** in 17 % yield. The ^{1}H NMR spectrum of **11** showed the characteristic singlets of the proton at the tertiary carbon in the lower half of the N4Py moiety at 5.35 ppm and the singlets of the methylenic protons from the top half at 3.93 and 3.94 ppm.[32] Furthermore, in the ^{13}C-NMR spectrum the signals of the methylenic carbons of the top half the and tertiary carbon in the lower half were observed at 56.89, 57.08 and 70.26 ppm, respectively, comparable to other N4Py derivatives.[32]
Because of the low yield of this alkylation step, combined with the number of steps still required to introduce the acridine (*e.g.* deprotection, conversion of the alcohol moiety to a primary amine followed by reaction with 9-chloro acridine), it was decided to abandon this route.

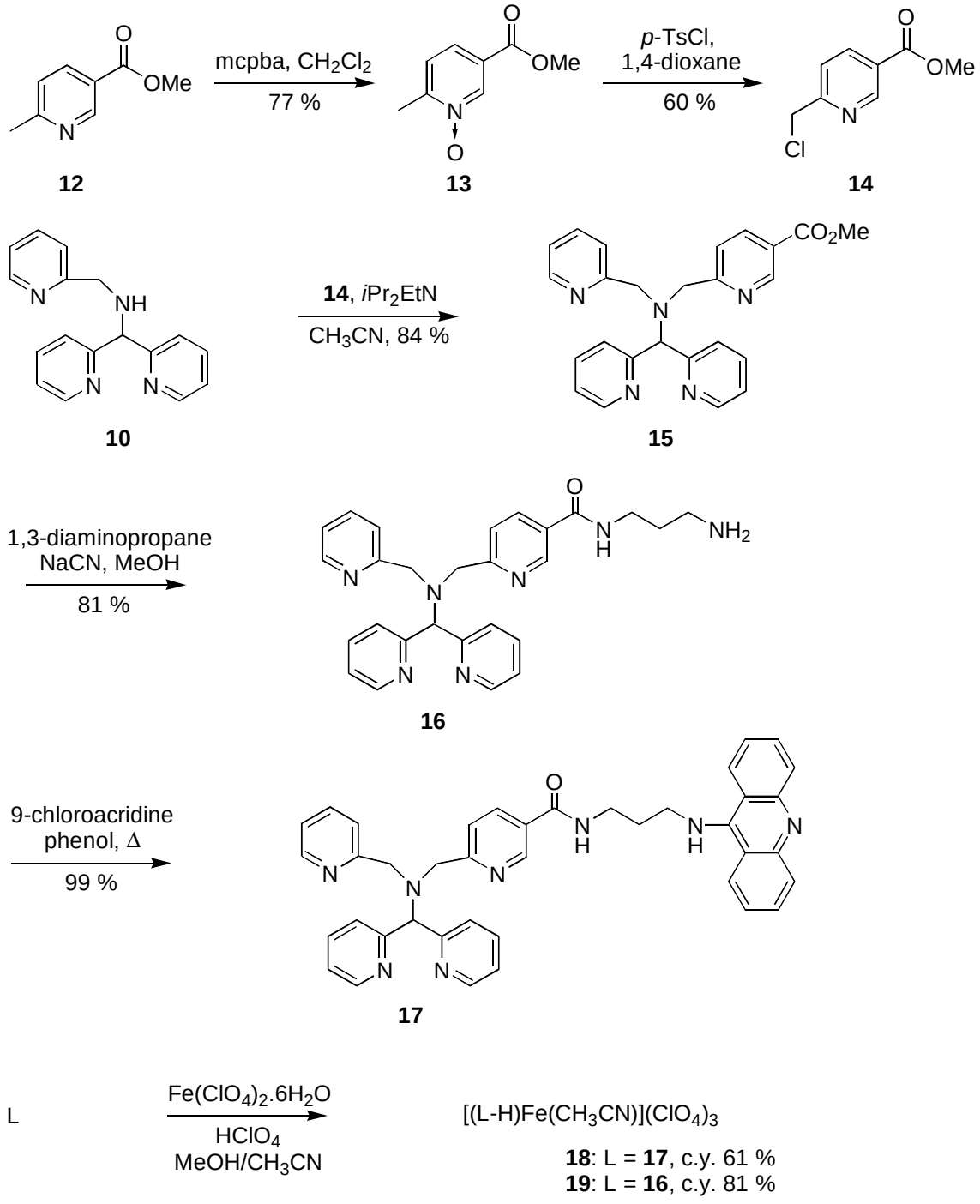


Scheme 5. *Synthetic approach A.*

7.8 Approach B

The key step in this approach is attachment of the spacer to an N4Py molecule via amide bond formation. For this purpose an N4Py with a carboxymethyl group was prepared from N3Py and methyl 6-(chloromethyl)nicotinate (**14**) (scheme 6). The latter was readily prepared starting from methyl 6-methylnicotinate (**12**) via *N*-oxide formation with mCPBA followed by direct conversion to the chloride **14** by reaction with *p*-toluenesulfonyl chloride in dioxane. The chloride was allowed to react with N3Py (**10**) in the presence of iPr$_2$EtN to give the N4Py derivative **15**. Reaction of **15** with 1,3-diaminopropane in the presence of a

catalytic amount of NaCN[35] resulted in the formation of the amide **16**. Finally, the acridine moiety was introduced by reaction of **16** with 9-chloro acridine in phenol to give the target ligand **17** in high yield. ^{1}H-NMR spectroscopy showed the characteristic singlet signals of an N4Py derivative from the protons at the methylenic carbons of the top half and the proton at the tertiary carbon of the lower half at 3.93, 4.00 and 5.31 ppm, respectively. Furthermore, the signals at 2.33, 3.69 and 4.23 ppm demonstrated the presence of the spacer and the characteristic acridine signals in the aromatic region were found at 8.45 ppm.



Scheme 6. *Synthesis of the target ligand and the corresponding iron complex.*

Metallation of **17** was carried out in CH_3OH/CH_3CN in the presence of 1 eq $HClO_4$ to protonate the very basic aminoacridine moiety, and [Fe(**17**-H)(CH_3CN)](ClO_4)$_3$·2H_2O (**18**) was obtained as slightly hygroscopic red crystals from CH_3CN/ethyl acetate. Its ^{1}H-NMR showed **18** to be a diamagnetic low-spin Fe(II) complex like [(N4Py)Fe(CH_3CN)](ClO_4)$_2$, and its protonated nature was established with ESI-MS, which exhibited signals at m/z 899 and 400, corresponding to {[(**17**-H)Fe](ClO_4)$_2$}$^+$ and {[(**17**-H)Fe](ClO_4)}$^{2+}$, respectively. The formulation of complex **18** was confirmed with elemental analysis. The complex of **16**, the precursor to ligand **17**, could be prepared similarly and was formulated as [(**16**-H)Fe(CH_3CN)](ClO_4)$_3$ (**19**), on the basis of its ^{1}H NMR and ESI-MS spectra, combined with elemental analysis.

7.9 DNA Cleavage

Figure 1a compares the cleavage of supercoiled Litmus29 plasmid DNA (0.1 μg/μL) by **1**, **18**, **19**, Fe-MPE, and Fe-BLM at 1 μM concentration under air *but with no added reductants*. The reactions were initiated by adding the metal complex to a buffered solution of DNA and quenched within 20 s by adding 10 μL of the solution to a solution of number III dye[30] and freezing with liquid nitrogen (figure 4, table 2).

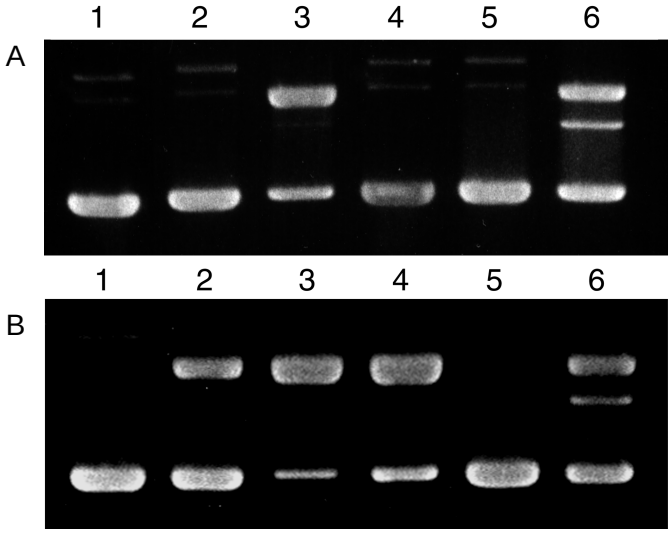


Figure 4. *Gels showing DNA cleavage with **18**, Fe-BLM and Fe-MPE at 1μM concentration; (a) immediately after addition, (b) after 1 h. Lane 1, DNA control; lane 2, complex **1**; lane 3, complex **18**; lane 4, complex **19**; lane 5, MPE-Fe^{II}; lane 6, BLM-Fe^{II}.*

Table 2, *Cleavage of the Litmus29 plasmid with 1 μM complex concentration.*[a]

reagent	time (h)	% form I[b]	% form II	% form III
Without DTT:				
Control	0	99	1	0
	1	99	1	0
1	0	99	1	0
	1	63	37	0
18	0	40	59	1
	1	25	74	1
19	0	98	2	0
	1	38	61	1
Fe^{II}-MPE	0	99	1	0
	1	97	3	0
Fe^{II}-BLM[c]	0	45	39	16
1 mM DTT:				
18[c]	0	0	85	15
Fe^{II}-MPE[c]	0	7	92	1
18[d]	0	62	38	0
	1	42	51	8
1 mM ascorbate:				
18[d]	0	38	56	7
	1	0	76	24

(a) Results not corrected for reduced ethidium bromide uptake capability of form I. (b) Corrected for not-nicked form II DNA. (c) Reliable quantification of DNA cleavage products at 1 h not possible because of DNA degradation. (d) At 0.1 μM complex concentration.

Immediately after addition significant DNA cleavage (>50%) was observed for **18** (figure 4a, lane 3) and Fe-BLM (figure 4a, lane 6), whereas **1**, **19**, and Fe-MPE showed less than 2% cleavage (lanes 2a, 4a, and 5a, respectively). Unlike Fe-BLM, which afforded both nicked and linear DNA, **18** produced only nicked DNA.[36] After 1 hour the amount of cleaved products had increased showing that the complex remains active after the initial burst of activity, but to a lower extent. Its cleavage activity was retained in the pH range from 5.7 to 9.2.[37] The enhanced cleavage activity of **18** is attributed to an increase in DNA affinity arising from the introduction of the DNA-intercalating acridine moiety. The electrostatic interaction between the cationic charge on the complex and the negatively charged phosphate backbone of the DNA may also play a role, as indicated by the higher activity of **19** compared to **1**. The contrast in the DNA cleavage activities of **18** and Fe-MPE in the absence of reducing agents is striking.

When reductants like DTT or ascorbate (0.01 – 1 mM) were added in the DNA cleavage experiments, significantly more cleavage activity was found. At 1 μM complex concentration and in the presence of 1mM DTT 85 % nicked and 15 % linear DNA was found for **18**,

compared to 92 % nicked and 1% linear for Fe-MPE. After 1 h many smaller DNA fragments are formed (degraded DNA) which makes quantification impossible. Due to this increased reactivity DNA cleavage could be obtained with as little as 0.1 μM **18**, giving 38 % nicked DNA immediately after addition and 51 % nicked and 8 % linear after 1 h. Under these conditions Fe-BLM is significantly more active than **18** and Fe-MPE. Using 1 mM ascorbate as reducing agent further increased the reactivity of **18**. Immediately after addition already 56 % nicked DNA was formed and 7 % linear DNA, and after 1 h all supercoiled DNA had disappeared and 76 % nicked DNA and 24 % linear DNA was formed.

7.10 ^{32}P End-Labeling Experiments

The nature of the termini of the DNA fragments was analyzed by 5'- ^{32}P end-labeling of a 183 bp restriction fragment. These experiments were performed both in the presence (figure 5a) and the absence of DTT (Figure 5b). The results were compared with DNaseI treatment, which produces 3'-hydroxyl termini (lane 2), the Maxam & Gilbert lanes (lanes 7 and 8), which show 3'-phosphate termini,[38] and Fe-BLM and Fe-MPE, which produce both 3'-phosphate and 3'-phosphoglycolate termini (lanes 3 and 4, respectively).[2,14,21]
The reaction of **18** with DNA yields two distinguishable DNA products (figure 5a, lane 6), which match well with the bands in the Fe-BLM lane and the Fe-MPE lane but do not match with those in the DNaseI lane. Similar results were obtained without DTT (figure 5b). These results show that DNA cleavage by **18** is oxidative in nature and not hydrolytic. Moreover, the production of 3'-phosphate and 3'-phosphoglycolate products are consistent with attack at the C4'-H of the deoxyribose, which is accessible through the minor groove. This result is fully consistent with the fact that 9-aminoacridine is known to intercalate into DNA and is able to direct tethered moieties into the minor groove.[39] The results after 1 h show that **18** cleaves DNA in an essentially sequence neutral manner. This result was expected since 9-amino acridines have been reported to intercalate with little sequence specificity.[26]

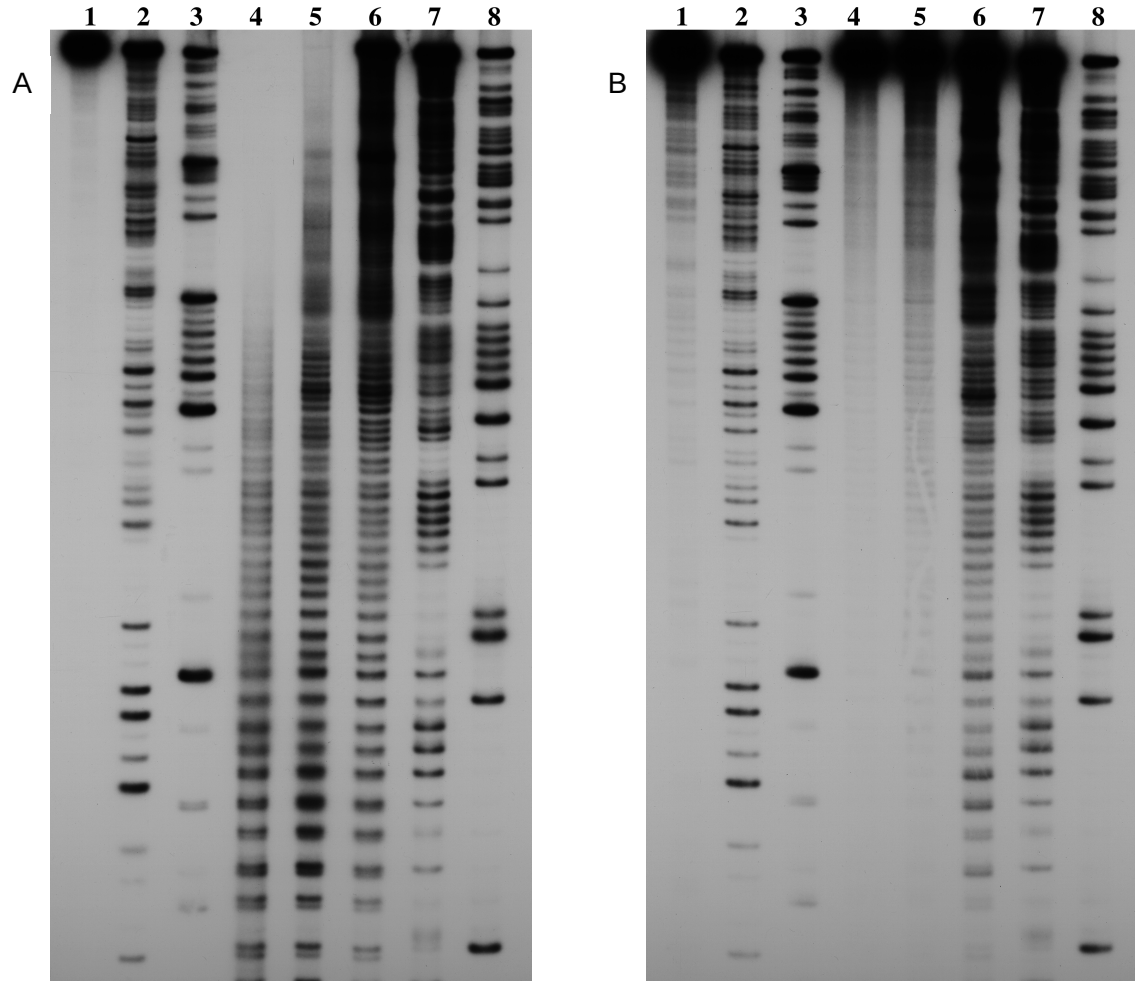


Figure 5. *High-resolution denaturing PAGE gels of cleavage products of a 5'-end-labeled 172-bp restriction fragment after 1h reaction. (a) In the presence of 1 mM DTT. Lane 1, control; lane 2, DnaseI; lane 3, BLM-Fe^{II} (10 μM); lane 4, MPE-Fe^{II} (10 μM); lane 5, Complex **18** (10 μM); lane 6,complex **18** (1 μM); lane 7, C & T; lane 8, G. (b) Without reducing agent. Lane 1, control; lane 2, DnaseI; lane 3, BLM-Fe^{II} (10 μM); lane 4, MPE-Fe^{II} (10 μM); lane 5, MPE-Fe^{II} (100 μM); lane 6,complex **18** (10 μM); lane 7, C & T; lane 8, G.*

However, the gel run after 24 hrs without reductant shows some "hot spots", *e.g.* some preferred areas of cleavage (Figure 6, lane 3). Some of the hot spots match with the Fe-BLM lane (lane 4), which has a preference for cleavage at GC base pairs, and others match well with the C&T lane (lane 1). No significant match with the G lane is found. From this it can be concluded that **18** shows a small preference for cleavage at C bases. Furthermore, careful analysis shows that these hot spots are not found in GC rich regions. More specific: complex **18** appears to prefer cleavage at C bases that are two nucleotides removed from a GT base stack (Figure 6b)

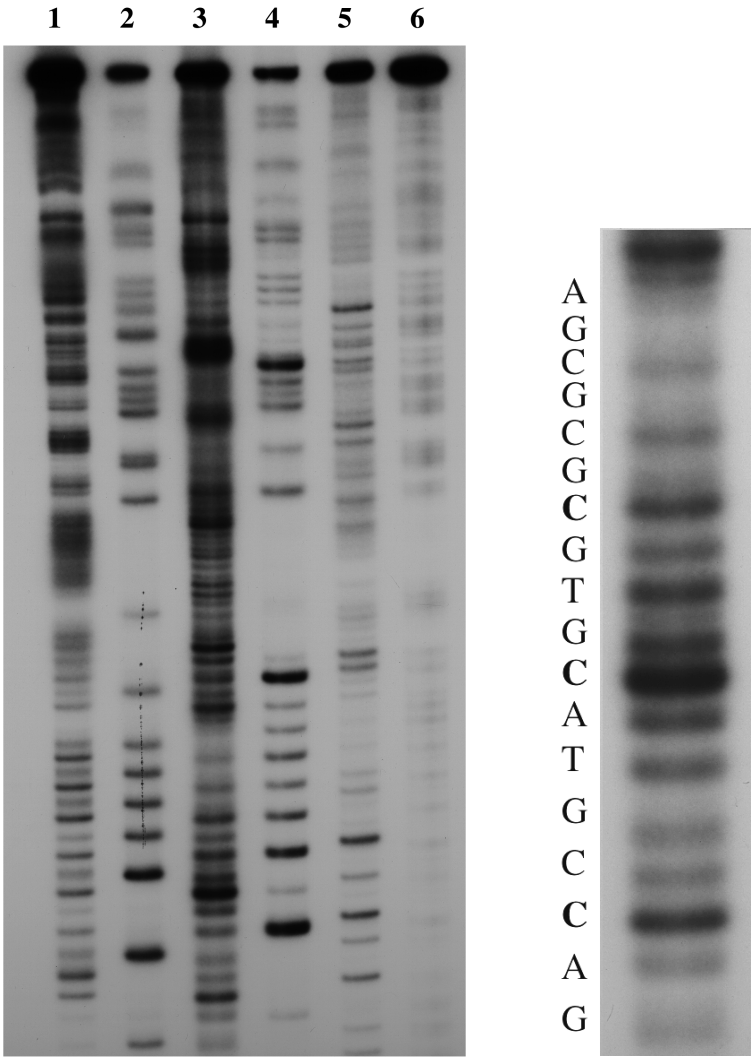


Figure 6. *(a) Analysis of base preference after 24 h without added reductant. Lane 1, C & T; lane 2, G; lane 3, complex **18** (10 μM); lane 4, BLM-Fe^{II} (10 μM) lane 5, DNaseI; lane 6, control. (b) Close up of lane 3 showing the base selectivity of DNA cleavage by **18**.*

The observed selectivity distinguished **18** from other synthetic iron containing DNA cleaving agents with covalently attached intercalating moieties like Fe-MPE,[21] which gives totally non selective DNA cleavage. However, it is difficult to compare the results of **18** with those of Fe-MPE since from the latter only data in the presence of reducing agent has been reported.[25] The origin of the preference for cleavage at C bases is unclear. The acridine should intercalate into the DNA in a sequence neutral manner, suggesting that the preference for C is caused by the metal binding (N4Py-Fe) part, similar to what is proposed for Fe-BLM.

7.11 Origin of the High Reactivity

The novel feature of **18** is that it can oxidatively cleave DNA instantaneously without added reductant, unlike FeMPE. The latter has a carboxylate-rich environment, which stabilizes the high-spin Fe^{III} state, and thus requires reductive activation. In contrast, **18** is an air-stable

low-spin Fe^{II} complex. This is presumably the result of the very high redox potential of 1090 mV vs. SCE observed for the Fe^{II}/Fe^{III} couple in CH_3CN. Upon dissolution in buffer, the coordinated CH_3CN is probably labilized and can be displaced by solvent water or the phosphodiester of the DNA, thereby converting the low-spin Fe^{II} center to a high-spin form that is capable of binding O_2 and activating it for DNA cleavage. This notion is supported by NMR studies of **1** and **18** in D_2O in the presence of buffer and/or DNA, which showed paramagnetic spectra with relatively sharp signals in the 0-80 ppm range, typical for a high-spin Fe^{II} complex.[40] However, at this time no conclusive answer can be given to the question what causes the high reactivity of the N4Py derived complexes **1**, **18** and **19**.

7.12 Conclusions

In conclusion, an N4Py iron complex that is covalently attached via a spacer to an acridine unit has been synthesized. This complex instantaneously cleaves DNA using O_2, without the necessity of adding external reducing agents. This feature makes **18** one of the most efficient synthetic DNA cleaving agents known to date. Since the initial cleavage is essentially sequence neutral this may lead to applications as DNA footprinting agent. Furthermore, introduction of more selective DNA binding units may lead to base pair or sequence selective DNA cleavage.

7.13 Experimental Section

General information

For general remarks see chapter 2. 5-bromo-2-picoline,[41] N3Py (**10**),[42] and 9-chloroacridine[43] were prepared according to literature procedures. The Litmus 29 plasmid DNA, prepared from DH5α cells, was purified using the Qiagen Plasmid Maxi Kit.

1-Bromo-6-(1-ethoxy-ethoxy)-hexane (3)

A solution of 6-bromohexan-1-ol (19.9 g, 93.4 mmol) in CH_2Cl_2 (250 ml) was cooled in an icebath. Ethyl vinyl ether (17 ml, 98.5 mmol) and a catalytic amount of pyridinium *p*-toluene sulfonate were added. After stirring for 2.5 h at 0 °C diethyl ether (750 ml) was added. The solution was washed with water (100 ml), brine (100 ml) and dried (Na_2SO_4). After removal of the solvent under reduced pressure the resulting liquid was vacuum distilled (0.1 mm, 74 °C) to give **3** (19.0 g, 70.6 mmol, 76 %) as a colorless liquid. ^{1}H-NMR ($CDCl_3$) δ 1.18 (t, 3H, J = 7.1 Hz), 1.27 (d, 3H, J = 5.4 Hz), 1.40 (m, 4H), 1.56 (m, 2H), 1.84 (m, 2H), 3.39 (t, 2H, J = 7.0 Hz), 3.44 (m, 4H), 4.55 (q, 1H, J = 5.4 Hz); ^{13}C NMR ($CDCl_3$) δ 15.21 (q), 19.75 (q), 25.37 (t), 27.88 (t), 29.59 (t), 32.64 (t), 33.34 (t), 60.63 (t), 64.92 (t), 99.52 (d).

6-(6-Methyl-pyridin-3-yl)-hexan-1-ol (4)

The Grignard reagent was prepared by carefully adding a solution of **3** (2.46 g, 9.7 mmol) in THF (13 ml) activated Mg (300 mg, 12.5 mmol) under an argon atmosphere. After reflux for 1 h the solution was transferred to a dropping funnel via cannula under argon. The Grignard reagent solution was added dropwise to a solution of 5-bromo-2-picoline (840 mg, 4.9 mmol)

and $NiCl_2$(dppp) (265 mg, 0.49 mmol) in THF (15 ml) at 0 °C. The reaction mixture was allowed to warm to room temperature overnight and then water (2 ml) was added. After evaporation of the solvent an aqueous HCl solution (0.2 N, 50 ml) was added. The water layer was washed with diethyl ether (2 × 50 ml), then made alkaline with NaOH and the product was extracted with diethyl ether (3 × 50 ml). The combined organic layers were washed with brine (30 ml), dried (Na_2SO_4) and the solvent removed *in vacuo*. Kugelrohr distillation (0.05 mmHg, 110 °C) afforded **4** (639 mg, 3.3 mmol, 68 %) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ 1.39 (m, 4H), 1.58 (m, 4H), 2.54 (s, 3H), 2.58 (t, 2H, J = 7.7 Hz), 3.62 (t, 2H, J = 6.6 Hz), 7.07 (d, 1H, J = 7.7 Hz), 7.34 (d, 1H,), 8.32 (s, 1H, J = 7.7 Hz); MS (CI): m/z 194 (M+1).

5-(6-Benzyloxy-hexyl)-2-methyl-pyridine (5)

To a suspension of NaH (465 mg, 11.6 mmol) in DMF (15 ml) was added dropwise a solution of **4** (1.47 g, 7.6 mmol) in DMF (20 ml). The mixture was heated at 50 °C for 2.5 h. After cooling to room temperature a solution of benzyl chloride (2.89 g, 22.8 mmol) in DMF (20 ml) was added and the reaction mixture was stirred overnight. To this mixture was added water (2 ml) and the solution was concentrated *in vacuo*. Addition of diethyl ether (50 ml) was followed by extraction with aqueous HCl (0.2 N, 3 × 20 ml). The combined aqueous layers were washed with diethyl ether (20 ml), made alkaline with NaOH and the product was extracted with diethyl ether (3 × 25 ml), the combined organic layers were washed with brine (20 ml), dried (Na_2SO_4) and concentrated *in vacuo*. Column chromatography (SiO_2, ethyl acetate/hexane 4:1) afforded **5** (1.54 g, 5.4 mmol, 72 %) as a slightly yellow oil. ^{1}H-NMR ($CDCl_3$) δ 1.38 (m, 4H), 1.60 (m, 4H), 2.52 (s, 3H), 2.56 (t, 2H, J = 7.7 Hz), 3.46 (t, 2H, J = 6.6 Hz), 4.50 (s, 2H), 7.07 (d, 1H, J = 7.7 Hz), 7.34 (m, 6H), 8.32 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 21.45 (q), 23.49 (t), 26.42 (t), 27.16 (t), 28.65 (t), 29.98 (t), 67.81 (t), 70.34 (t), 120.25 (d), 124.96 (d), 125.07 (d), 125.82 (d), 132.08 (s), 133.65 (d), 136.14 (s), 146.60 (d), 153.02 (s); MS (CI): m/z 284 (M+1).

5-(6-Benzyloxy-hexyl)-2-methyl-pyridine *N*-oxide (6)

A solution of **5** (1.54 g, 5.4 mmol) and *m*-chloroperoxybenzoic acid (1.95 g, 6.8 mmol) in CH_2Cl_2 (20 ml) was stirred overnight at room temperature. Saturated aqueous $NaHCO_3$ (20 ml) was added and the mixture was stirred for 2 h. The CH_2Cl_2 layer was separated and the aqueous layer was extracted with CH_2Cl_2 (3 × 20 ml). The combined organic layers were washed with water (20 ml) and brine (20 ml), dried (Na_2SO_4) and the solvent removed *in vacuo* to afford **6** (1.91 g, 5.38 mmol, 99 %) as a colorless liquid. ^{1}H-NMR ($CDCl_3$) δ 1.34 (m, 4H), 1.59 (m, 4H), 2.47 (s, 3H), 2.51 (t, 2H, J = 7.3 Hz), 3.44 (t, 2H, J = 6.4 Hz), 4.48 (s, 2H), 7.00 (dd, 1H, J = 8.1 Hz, J = 1.5 Hz), 7.15 (d, 1H, J = 8.1 Hz), 7.31 (m, 5H), 8.12 (s, 1H); MS (CI): m/z 300 (M+1).

Acetic acid 5-(6-benzyloxy-hexyl)-pyridin-2-ylmethyl ester (7)

Excess acetic anhydride (10 ml) was added to **6** (1.61 g, 5.7 mmol) and the mixture was heated overnight at 100 °C. After cooling to room temperature the mixture was poured out in water and made alkaline with Na_2CO_3 followed by extraction with ethyl acetate (3 × 20 ml).

The combined ethyl acetate layers were washed with brine (20 ml), dried (Na_2SO_4) and concentrated *in vacuo* to afford **7** (1.7 g, 5.0 mmol, 88 %) as a dark liquid which was used without further purification. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.39 (m, 4H), 1.61 (m, 4H), 2.15 (s, 3H), 2.60 (t, 2H, J = 8.1 Hz), 3.46 (t, 2H, J = 6.4 Hz), 4.49 (s, 2H), 5.18 (s, 2H), 7.33 (m, 6H), 7.48 (dd, 1H, J = 8.1 Hz, J = 2.0 Hz), 8.41 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 20.71 (q), 25.73 (t), 28.68 (t), 29.38 (t), 30.79 (t), 32.39 (t), 66.68 (t), 70.09 (t), 72.66 (t), 121.65 (d), 127.36 (d), 127.47 (d), 128.21 (d), 136.44 (d), 137.15 (s), 138.47 (s), 149.55 (d), 152.81 (s), 170.70 (s); MS (CI): m/z 342 (M+1).

(5-(6-Benzyloxy-hexyl)-pyridin-2-yl)-methanol (8)

To a solution of **7** (1.67 g, 4.9 mmol) in methanol (20 ml) was added K_2CO_3 (700 mg, 5.0 mmol). The suspension was stirred for 1 h at room temperature followed by removal of the solvent under reduced pressure. Water (20 ml) was added and the product was extracted with ethyl acetate (3 × 20 ml). The combined ethyl acetate layers were washed with brine (15 ml), dried (Na_2SO_4) and concentrated *in vacuo* to afford **8** (1.35 g, 4.5 mmol, 92 %) as a slightly yellow oil. ^{1}H-NMR ($CDCl_3$) δ 1.37 (m, 4H), 1.61 (m, 4H), 2.60 (t, 2H, J = 7.3 Hz), 3.46 (t, 2H, J = 6.4 Hz), 4.94 (s, 2H), 4.73 (s, 2H), 7.18 (d, 1H, J = 7.8 Hz), 7.33 (m, 5H), 7.47 (dd, 1H, J = 7.8 Hz, J = 1.7 Hz), 8.36 (s, 1H).

5-(6-Benzyloxy-hexyl)-2-chloromethyl-pyridine (9)

A solution of **8** (1.34 g, 4.5 mmol) in CH_2Cl_2 (15 ml) is added dropwise to a solution of $SOCl_2$ (1 ml, 13.5 mmol) in 5 ml CH_2Cl_2. After stirring overnight saturated aqueous $NaHCO_3$ is carefully added. The CH_2Cl_2 layer was separated and the aqueous layer extracted with CH_2Cl_2 (3 × 15 ml). The combined organic layers were washed with brine (20 ml), dried (Na_2SO_4) and concentrated *in vacuo* to afford **9** (1.36 g, 4.3 mmol, 96 %) as a dark liquid, which was used without further purification. ^{1}H-NMR ($CDCl_3$) δ 1.39 (m, 4H), 1.61 (m, 4H), 2.61 (t, 2H, J = 7.3 Hz), 3.46 (t, 2H, J = 6.4 Hz), 4.50 (s, 2H), 4.65 (s, 2H), 7.33 (m, 6H), 7.50 (dd, 1H, J = 8.1 Hz, J = 2.2 Hz), 8.40 (d, 1H, J = 1.7 Hz); ^{13}C NMR ($CDCl_3$) δ 25.73 (t), 28.68 (t), 29.38 (t), 30.76 (t), 32.38 (t), 46.41 (t), 70.09 (t), 72.68 (t), 122.38 (d), 127.37 (d), 127.48 (d), 128.22 (d), 136.80 (d), 137.39 (s), 138.47 (s), 149.42 (d), 153.77 (s).

(5-(6-Benzyloxy-hexyl)-pyridin-2-ylmethyl)-(di-pyridin-2-ylmethyl)-pyridin-2-ylmethyl-amine (11)

A solution of N3Py (100 mg, 0.36 mmol), **9** (120 mg, 0.40 mmol), K_2CO_3 (50 mg, 0.36 mmol) and a catalytic amount of NaI in CH_3CN (5 ml) was heated under reflux overnight under an argon atmosphere. After cooling to room temperature the solvent was removed under reduced pressure. Water (10 ml) was added and the product was extracted with diethyl ether (3 × 20 ml). The combined organic layers were washed with brine (10 ml), dried (Na_2SO_4) and concentrated *in vacuo*. Column chromatography (Alox neutral akt. I, ethyl acetate/hexane/triethylamine 10:5:1) afforded **11** (35 mg, 0.06 mmol, 17 %) as a yellow oil. ^{1}H-NMR ($CDCl_3$) δ 1.39 (m, 4H), 1.58 (m, 4H), 2.55 (t, 2H, J = 7.5 Hz), 3.46 (t, 2H, J = 6.4 Hz), 3.93 (s, 2H), 3.97 (s, 2H), 4.50 (s, 2H) 5.35 (s, 1H), 7.15 (m, 3H), 7.33 (m, 5H), 7.51 (m, 2H), 7.71 (m, 6H), 8.32 (d, 1H, J = 1.5 Hz), 8.51 (m, 1H), 8.56 (m, 2H); ^{13}C NMR ($CDCl_3$) δ

25.87 (t), 28.88 (t), 29.54 (t), 31.00 (t), 33.00 (t), 56.89 (t), 57.08 (t), 70.26 (t), 71.86 (d), 72.78 (t), 121.71 (d), 122.03 (d), 122.47 (d), 122.83 (d), 123.93 (d), 127.48 (d), 127.61 (d), 128.34 (d), 135.87 (s), 136.27 (d), 138.63 (s), 149.03 (d), 149.10 (d), 149.28 (d), 156.98 (s), 190.00 (s), 190.06 (s).

Methyl 6-methylnicotinate *N*-oxide (13)

Methyl 6-methylnicotinate (10 g, 66.2 mmol) was dissolved in dichloromethane (150 ml). 3-Chloroperoxybenzoic acid (17 g, 112 mmol) was added and the mixture was stirred for 3 h at room temperature. Saturated $NaHCO_3$ solution (200 ml) was added and the mixture was stirred for an additional hour. The dichloromethane layer was separated and the aqueous layer was extracted with dichloromethane (2 × 100 ml). The combined dichloromethane layers were washed with saturated $NaHCO_3$ (aq) (100 ml), brine (100 ml) and dried (Na_2SO_4). After evaporation of the solvent **13** (7.8 g, 51.0 mmol, 77 %) was obtained as a creme colored solid, mp 90.4 – 90.8 °C. ^{1}H-NMR ($CDCl_3$) δ 2.52 (s, 3H), 3.90 (s, 3H), 7.32 (d, 1H, J = 8.05 Hz), 7.70 (dd, 1H, J = 8.05 Hz, J = 1.1 Hz), 8.80 (d, 1H, J = 1.1 Hz); HRMS calcd for $C_8H_9NO_3$ 167.058, found 167.060.

Methyl 6-(chloromethyl)nicotinate (14)

p-Toluenesulfonyl chloride (10.7 g, 56.1 mmol) was combined with **13** (7.8 g, 51.0 mmol) in dioxane (100 ml) under an Argon atmosphere. The reaction mixture was heated under reflux for 1 night. After cooling to room temperature the solvent was evaporated and the residue dissolved in dichloromethane (200 ml). The solution was washed with saturated Na_2CO_3 (aq) (2 × 100 ml), brine (50 ml) and dried (Na_2SO_4). After evaporation of the solvent the product was purified by column chromatography (SiO_2, hexane/ethyl acetate 10:2.5) to give **14** (5.71 g, 30.8 mmol, 60 %) as a slightly yellow solid. An analytically pure sample could be obtained by recrystallization from *n*-hexane, mp 63.5 – 63.8 °C. ^{1}H-NMR ($CDCl_3$) δ 3.94 (s, 3H), 4.70 (s, 2H), 7.58 (d, 1H, J = 8.4 Hz), 8.30 (dd, 1H, J = 8.1 Hz, J = 2.2 Hz), 9.08 (d, 1H, J = 1.5 Hz); Anal. calcd for $C_8H_8ClNO_2$: C 51.77, H 4.34, N 7.55; found: C 51.50, H 4.23, N 7.46.

6-(((di-pyridin-2-yl-methyl)-pyridin-2-ylmethyl-amino)-methyl) nicotinic acid methyl ester (15)

A solution of N3Py (1.45 g, 5.3 mmol), **14** (1.08 g, 5.8 mmol) and *N*,*N*-diisopropylethylamine (1.3 ml, 7.5 mmol) in acetonitrile (20 ml) was heated under reflux overnight, under an Argon atmosphere. After cooling to room temperature the solvent was evaporated and the residue was purified by column chromatography (Al_2O_3 neutral akt. I, ethyl acetate/triethylamine 10:1) to give **15** (1.96 g, 84 %) as a dark oil. ^{1}H-NMR ($CDCl_3$) δ 3.91 (s, 3H), 3.95 (s, 2H), 4.05 (s, 2H), 5.33 (s, 1H), 7.11 (m, 3H), 7.65 (m, 7H) 8.20 (m, 1H), 8.48 (d, 1H, J = 4.9 Hz), 8.56 (d, 2H, J = 4.9 Hz), 9.06 (d, 1H, J = 2.2 Hz); ^{13}C NMR ($CDCl_3$) δ 52.17 (q), 57.18 (t), 57.56 (t), 72.31 (d), 121.91 (d), 122.19 (d), 122.21 (d), 122.39 (d), 123.04 (d), 123.93 (d), 124.06 (s), 136.33 (d), 137.32 (d), 149.12 (d), 149.34 (d), 150.20 (d), 159.42(s), 159.82 (s), 164.97 (s), 166.13 (s); MS (CI): m/z 426 (M+1).

_N_-(3-amino-propyl)-6-(((di-pyridin-2-yl-methyl)-pyridin-2-ylmethyl-amino)-methyl) nicotinamide (16)

A solution of **15** (473 mg, 1.11 mmol), 1,3-diaminopropane (1.1 ml, 13.1 mmol) and NaCN (7 mg, 0.14 mmol) in methanol (15 ml) was heated under reflux for 24 hours under an Argon atmosphere. After cooling to room temperature the mixture was poured into water (100 ml) and the aqueous layer was washed with ether (2 × 125 ml), followed by extraction with dichloromethane (3 × 75 ml). The combined dichloromethane layers were washed with water (50 ml), brine (50 ml) and dried (Na_2SO_4). Evaporation of the solvent afforded **16** (418 mg, 81%) as a slightly yellow sticky solid. ^{1}H-NMR ($CDCl_3$) δ 1.71 (m, 2H), 2.91 (m, 2H), 3.54 (m, 2H), 3.91 (s, 2H), 3.96 (s, 2H), 5.29 (s, 1H), 7.09 (m, 3H), 7.60 (m, 7H), 8.03 (m, 1H), 8.43 (d, 1H, J = 4.4 Hz), 8.52 (d, 2H, 4.8 Hz), 8.85 (s, 1H); ^{1}H-NMR ($CDCl_3$) δ 30.41 (t), 39.34 (t), 40.51 (t), 56.79 (t), 57.14 (t), 71.86 (d), 121.81(d), 122.07 (d), 122.35 (d), 122.86 (d), 123.82 (d), 128.36 (s), 135.39 (d), 136.23 (d), 136.29 (d), 147.42 (d), 148.95 (d), 149.18 (d), 159.27 (s), 159.58 (s), 162.58 (s), 165.33 (s); MS (CI): m/z 468 (M+1).

_N_-(3-(acridin-9-ylamino)-propyl)-6-(((di-pyridin-2-ylmethyl)-pyridin-2-ylmethyl-amino)-methyl) nicotinamide (17)

A mixture of **16** (301 mg, 0.64 mmol), 9-chloroacridine (140 mg, 0.64 mmol) and phenol (1.5 g) was heated at 90 °C during 3 hours under an Argon atmosphere. After cooling to room temperature diethyl ether was added (50 ml) and the suspension was stirred for 30 min. The diethyl ether was decanted, fresh diethyl ether was added (20 ml) and the suspension was stirred for 15 min after which the diethyl ether was decanted. 1 N NaOH (50 ml) was added and the aqueous layer was extracted with dichloromethane (3 × 75 ml). The combined dichloromethane layers were washed with brine (50 ml), dried (Na_2SO_4) and the solvent removed under reduced pressure. The solid residue was dissolved in ethanol (15 ml) and dry HCl gas was bubbled through the solution for 30 min. Diethyl ether (10 ml) was added and the hygroscopic precipitate filtered under argon. 1 N NaOH (50 ml) was added to the salt and the aqueous layer was extracted with dichloromethane (3 × 50 ml). The combined dichloromethane layers were washed with brine (50 ml), dried (Na_2SO_4) and the solvent removed *in vacuo* to give **17** (410 mg, 0.63 mmol, 99 %) as a yellow solid, mp >250 °C. ^{1}H-NMR ($CDCl_3$) δ 2.00 (m, 2H), 3.67 (m, 2H), 3.90 (m, 2H), 3.93 (s, 2H), 4.00 (s, 2H), 5.31 (s, 1H), 7.19 (m, 5H), 7.31 (m, 2H), 7.61 (m, 9H), 7.99 (m, 1H), 8.04 (m, 2H), 8.22 (d, 2H, J = 8.2 Hz), 8.47 (d, 1H, J = 4.4 Hz), 8.54 (d, 2H, J = 4.8 Hz), 8.85 (s, 1H); ES/MS: m/z 645 (M+1).

[(17-H)Fe(CH_3CN)](ClO_4)$_2$·2H_2O (18)

A solution of 70 % perchloric acid (26 mg, 0.18 mmol) in methanol (2 ml) was added to **17** (116 mg, 0.18 mmol). To this solution was added Fe(ClO_4)$_2$·6H_2O (72 mg, 0.2 mmol) in acetonitrile (2 ml). The dark red solution was divided in three equal portions and placed in an ethyl acetate bath. After 1 night **18** was obtained as a red solid which was recrystallized by slow vapor diffusion of ethyl acetate into an acetonitrile solution of **18**. After 3-4 days **18** (115 mg, 61 %) was obtained as red crystals. ^{1}H-NMR (CD_3CN) δ 2.33 (m, 2H), 3.69 (m, 2H), 4.23 (m, 2H), 4.44 (m, 4H), 6.34 (s, 1H), 7.10 (d, 1H, J = 8.1 Hz), 7.14 (d, 1H, J = 8.1

Hz),7.36 (m, 2H), 7.49 (t, 1H, J = 7.3 Hz), 7.70 (dt, 1H, J = 7.7 Hz, J = 1.1 Hz), 7.78 (d, 1H, J = 8.4 Hz), 7.89 (m, 7H), 8.45 (d, 2H, J = 8.8 Hz), 8.90 (d, 1H, J = 5.1 Hz), 9.01 (d, 1H, J = 5.5 Hz), 9.05 (br, 1H), 9.18 (d, 1H, J = 5.5 Hz), 9.24 (d, 1H, J = 1.1 Hz), 11.19 (br, 1H); ES/MS: m/z 899 $[M - (ClO_4)^- - (CH_3CN)]^+$, 400 $[M - 2(ClO_4)^- - (CH_3CN)]^{2+}$; Anal. calcd for $C_{42}H_{44}Cl_3Fe_1N_9O_{15}$: C 46.84, H 4.12, N 11.70; found: C 46.99, H 4.17, N 11.70.

$[(16\text{-}H)Fe(CH_3CN)](ClO_4)_2$ (19)

To a solution of **16** (97 mg, 0.21 mmol) in CH_3CN (4 ml) was added 70 % perchloric acid (32 mg, 0.22 mmol), followed by $Fe(ClO_4)_2 \cdot 6H_2O$ (82 mg, 0.22 mmol). The solution was placed in an ethyl acetate bath and after 3 days **19** (146 mg, 0.17 mmol, 81 %) was isolated as red crystals. ^{1}H-NMR (CD_3CN) δ 1.98 (m, 2H), 3.07 (m, 2H), 3.52 (m, 2H), 4.36 (m, 4H), 6.33 (s, 1H), 7.06 (d, 1H, J = 7.7 Hz), 7.14 (d, 1H, J = 8.4 Hz), 7.32 (m, 3H), 7.67 (dt, 1H, J = 7.7 Hz, J = 1.1 Hz), 7.97 (m, 6H), 8.89 (d, 1H, J = 5.1 Hz), 9.01 (d, 1H, J = 5.5 Hz), 9.17 (d, 1H, J = 5.1 Hz), 9.21 (d, 1H, J = 1.1 Hz); ES/MS: m/z 722 $[M - (ClO_4)^- - (CH_3CN)]^+$, 311.5 $[M - 2(ClO_4)^- - (CH_3CN)]^{2+}$; Anal. calcd for $C_{29}H_{33}Cl_3Fe_1N_8O_{13}$: C 40.37, H 3.87, N 13.00; found: C 40.26, H 3.86, N 13.05.

DNA cleavage experiments, typical procedure

A solution of metal complex was added to a buffered (Tris, 10 mM, pH 8.0) solution of Litmus29 plasmid DNA (0.1 μg/μl) at 37 °C. When reducing agents were used they were always added last. At indicated times a sample (10 μl) was taken from the reaction and quenched with a solution of number III dye[30] (2 μl, consisting of 0.04 % bromophenol blue, 0.04 % xylene cyanol FF, and 5 % glycerol) and immediately frozen in liquid nitrogen. All gels were run on 1.2 % agarose slab gels for 120 min at 70 V. Gels were stained with ethidium bromide and pictures taken with Polaroid 3000 type 667 film. The intensities of the bands on the film were quantified using the software program scion image 3b.

End group analysis

The 5'-end-labeled restriction fragments were prepared by treatment of Litmus29 plasmid DNA with 50 U of *Hind*III followed by ethanol precipitation. The DNA was treated with 3 U of calf intestinal phosphatase followed by heat treatment at 75 °C for 10 min in the presence of 10 mM pH 8 EDTA. After phenol-chloroform-isoamyl alcohol treatment and ethanol precipitation the DNA was 5'-end-labeled with 2 U T4 polynucleotide kinase in the presence of [γ-^{32}P]-ATP. The DNA was treated with phenol-chloroform-isoamyl alcohol and run through a Sephadex G-50 column equilibrated to pH 8 with Tris buffer. After NH_4OAc/ethanol precipitation, the DNA was treated with 4 U of *Pvu*II and purified with a 6 % nondenaturing PAGE gel. DNA was visualized by exposure to x-ray film and purified from the gel using the crush and soak method.[30]

The 5'-end-labeled restriction fragments (800,000-1,000,000 cpm) were treated with the cleavage agent at the indicated concentration in the presence of 0.25 μg/10 μl unlabeled linear fragments of Litmus 29 in Tris buffer (10 mM, pH 8) in a total volume of 100 μl. Where necessary DTT was added to give a final concentration of 1mM. After the incubation for the indicated times the samples were ethanol precipitated and suspended in 20 μl H_20.

Maxam and Gilbert reactions followed standard microbiology protocols.[30] DNase reactions were carried out in Tris (10 mM) for 15 min with serial dilutions of DNase I. The samples were run on a 20 % denaturing PAGE 7 M urea gel for 210 min at 50 V, then exposed to Fuji RX x-ray film.

7.14 References and Notes

1 Abbrevations used: BLM = bleomycin; ssb = single strand breaks; dsb = double strand breaks; EDTA = *N*,*N*,*N*',*N*'-ethylenediaminetetraacetate; MPE = methidium propyl EDTA; N4Py = *N*,*N*-bis(2-pyridylmethyl)-*N*-bis(2-pyridyl)methylamine; dppp = 1,3-bis(diphenylphosphino)propane; EE = ethoxy ethyl; N3Py = *N*-[di(2-pyridinyl)methyl]-*N*-(2-pyridinylmethyl)amine; DTT = dithiothreitol.

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